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SAHA and S116836, a novel tyrosine kinase inhibitor, synergistically induce apoptosis in imatinib-resistant chronic myelogenous leukemia cells

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Keywords: CML, Bcr-Abl, T315I mutation, tyrosine kinase inhibitor, imatinib, resistance, S116836, apoptosis

Abbreviations: CML, chronic myelogenous leukemia; IM, imatinib mesylate; HDACs, histone deacetylases; HDACi, HDAC inhibitor; SAHA, suberoylanilide hydroxamic acid

Limited treatment options are available for chronic myelogenous leukemia (CML) patients who develop imatinib mesylate (IM) resistance. Here we proposed a novel combination regimen, a co-administration of S116836, a novel small molecule multi-targeted tyrosine kinase inhibitor that was synthesized by rational design, and histone deacetylases inhibitor (HDACi) suberoylanilide hydroxamic acid (SAHA), to overcome IM resistance in CML. S116836 at low concentrations used in the present study mildly downregulates auto-tyrosine phosphorylation of Bcr-Abl. SAHA, an FDA-approved HDACi drug, at 1 μM has modest anti-tumor activity in treating CML. However, we found a synergistic interaction between SAHA and S116836 in Bcr-Abl-positive CML cells that were sensitive or resistant to IM. Exposure of KBM5 and KBM5-T315I cells to minimal or non-toxic concentrations of SAHA and S116836 synergistically reduced cell viability and induced cell death. Co-treatment with SAHA and S116838 repressed the expressions of anti-apoptosis proteins, such as Mcl-1 and XIAP, but promoted Bim expression and mitochondrial damage. Of importance, treatment with both drugs significantly reduced cell viability of primary human CML cells, as compared with either agent alone. Taken together, our findings suggest that SAHA exerts synergistically with S116836 at a non-toxic concentration to promote apoptosis in the CML, including those resistant to imatinib or dasatinib.

Introduction

Chronic myelogenous leukemia (CML) is a type of hematopoietic stem cell disorder arising from chromosomal aberration between chromosomes 9 and 22.[1] This chromosomal abnormality, known as the Philadelphia chromosome, leads to myeloproliferation.[2] The balanced and reciprocal translocation of the chromosomes results in the creation of Bcr-Abl gene, which encodes a Bcr-Abl protein with enhanced tyrosine kinase activity.[3] Bcr-Abl is able to activate a wide range of signaling pathways. For example, Bcr-Abl increases the activation and/or expression of a series of anti-apoptotic proteins such as STATs,[4] Akt,[5] PI3K,[6] Mcl-1,[7] and Bcl-X_L.[8] Imatinib is a well-established small molecule tyrosine kinase inhibitor that specifically targets the ATP-binding site of Bcr-Abl to prevent the autophosphorylation of Bcr-Abl itself.[9] Despite the specific and remarkable effect of imatinib, an increasing number of CML patients resistant to imatinib are emerging in clinic.[10] A frequent cause of the imatinib-resistance is point mutations in the Bcr-Abl relevant domains. There are more than 100 reported mutations[11-13] of which most can be conquered by the second-generation tyrosine kinase inhibitors (e.g., nilotinib, dasatinib), with the exception of the gate-keeper mutation T315I.[14-16] Ponatinib, a third-generation of tyrosine kinase inhibitor, has shown activity against refractory CML patients including those harboring T315I Bcr-Abl. However, the long-term benefit of ponatinib has to be balanced against the risk of deleterious side effects in these patients.

S116836 was originally designed and synthesized against T315I Bcr-Abl (**Fig. 1A**). The kinase assay showed that it blocked multiple tyrosine kinases including both wild-type

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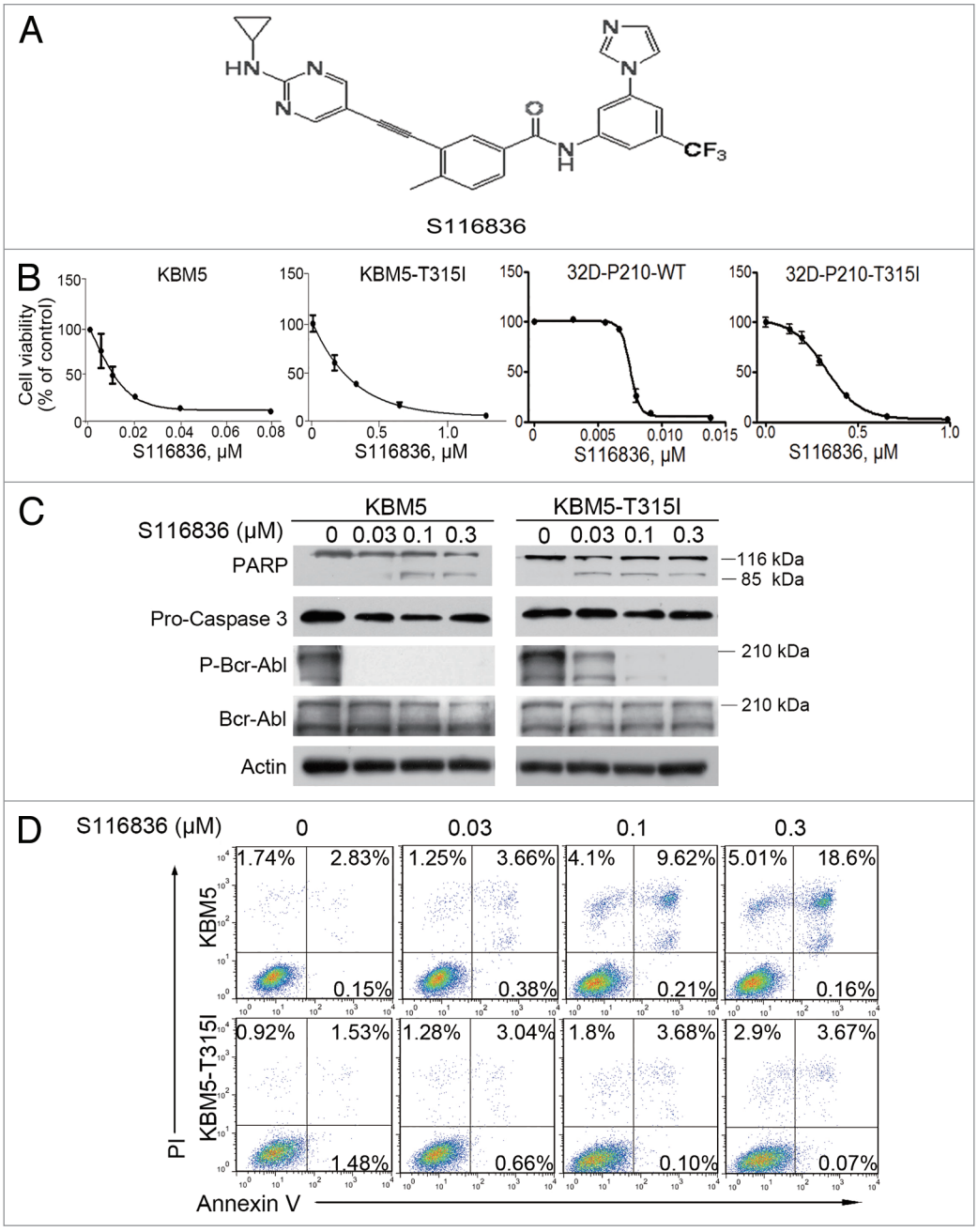

Figure 1. Treatment with S116836 inhibits growth and induces apoptosis of chronic myelogenous leukemia (CML) cells. (**A**) Chemical structure of compound S116836. (**B**) Cells were treated with S116836 for 72 h, cells viability was evaluated by using MTS assay. (**C**) KBM5 or KBM-T315I cells were exposed to increasing concentrations of S116836 for 24 h; western blotting analysis was performed with the indicated antibodies. (**D**) The CML cells were exposed to escalating concentrations of S116836 for 24 h, the percentages of apoptotic cells were detected by flow cytometry after dual labeling with FITC-Annexin V and propidium iodide (PI).

as well as T315I Bcr-Abl. In addition, S116836 showed potent inhibitory effect on the SRC family kinases SRC, LYN, HCK, LCK, and BLK, and receptor tyrosine kinase such as FLT3, TIE2, KIT, PDGFRβ (Pan J et al., unpublished data). The lesson taken from imatinib and the second-generation of small molecule inhibitors (nilotinib and dasatinib) is that resistance will most likely arise after attenuating the function of one target. Disrupting a single pathway will likely be insufficient to eliminate the abnormal myeloid cells. The lack of efficacious and safe therapeutic regimen for the patients with cross-resistance to imatinib and dasatinib calls for a novel therapeutic strategy. The administration of multiple drugs simultaneously, as compared with single agent, exhibits greater anti-tumor activities and overcomes the drug resistance problems. It has been reported that combinations of histone deacetylase inhibitor (HDACi) with a series of small molecule inhibitors show synergistic effects in inducing apoptosis of various cancer cell lines.[17-19]

Histone acetylation plays a pivotal role in the regulation of gene expression. Histone acetyltrasferases transfer the acetyl moieties to the lysine residues of histones to form a relaxed chromatin state. In contrast, histone deacetylases (HDACs) remove the acetyl modification from lysine residues of histone, leading to a condensed chromatin state.[20] The balance between acetylation and deacetylation of the chromatin serves as a key epigenetic mechanism for transcription factor-dependent gene expression, which are consequently crucial for numerous fundamental cellular processes including cell cycle,[21] apoptosis,[22] DNA repair,[23] and differentiation.[24] However, the abnormal recruitments of HDAC to the promoter of the anti-tumor genes are closely associated with the onset and progression of tumor.[25-29] HDACi have been shown to display anti-tumor ability by triggering apoptosis,[30] inducing differentiation,[31] suppressing cell proliferation,[32] and arresting cell cycle.[33,34] An additional advantage of HDACi is their low toxicity to the normal tissues.[35]

Suberoylanilide hydroxamic acid (SAHA), also known as vorinostat, is the first HDACi approved by US FDA for the treatment of cutaneous manifestations in patients with T-cell lymphoma.[36] SAHA represses HDACs by directly binding to the catalytic domain of the HDACs, ultimately causing overexpression of pro-apoptosis proteins (e.g., BAX, BAK, and Bim),[37,38] and reduced expression of the anti-apoptosis proteins such as XIAP,[39] Bcl-2,[40] Bcl-X_L,[41] and Mcl-1.[42] In addition, SAHA is able to raise the level of reactive oxygen species and promote the acetylation of non-histone proteins (e.g., p53 and heat shock protein 90, Hsp90).[43-45] When HDAC6 is inhibited by SAHA treatment, the chaperone function of Hsp90 was shown to be disrupted through acetylation itself,[46] which finally results in the depletion of Bcr-Abl, Akt, and c-Raf. It is of interest that SAHA can work synergistically with various drugs, including the tyrosine kinase inhibitor (imatinib, dasatinib),[47] Hsp90 antagonist (7-AAG),[48] MEK1/2 inhibitor (PD184352),[49] and aurora kinase inhibitor (MK-0457),[50] to induce apoptosis of imatinib-resistant leukemia cells.

We hypothesized that SAHA had synergistic effect with S116836 on T315I Bcr-Abl CML cells. This study was to test the hypothesis. We discovered that combination of SAHA and S116836 has synergistic anti-leukemic activity in both wild-type and T315I Bcr-Abl CML cells. Our findings supported that combination of SAHA and S116836 hold promise to overcome imatinib-resistance in CML cells.

Results

S116836 inhibits cell growth and induces apoptosis in imatinib-sensitive and imatinib-resistant CML cells

S116836 is a novel compound to inhibit tyrosine kinase activity (**Fig. 1A**). To determine its effect on CML cells, we first investigated the cell viability of KBM5 and KBM5-T315I under the treatment of S116836. Cells were cultured with escalating concentrations of S116836 for 72 h. MTS assay were performed to detect cell viability. S116836 inhibited growth in KBM5 and KBM5-T315I in a dose-dependent fashion with IC_{50} values of 15.73 and 407.96 nM, respectively (**Fig. 1B**). Similarly, S116836 dose-dependently inhibited growth in 32D-P210-WT and 32D-P210-T315I with IC_{50} values of 7.1 and 362 nM, respectively (**Fig. 1B**).

S116836 at lower concentrations (~0.3 μM) appreciably blocked the phosphorylation of Bcr-Abl, but induced limited apoptosis in CML cells, particularly in imatinib-resistant KBM5-T315I, which was supported by the limited PARP cleavage and Annexin V-positive cells (**Fig. 1C and D**).

Combination of SAHA and S116836 induces increased acetylated p53 and blockade of Bcr-Abl in CML cells

Insurgence of resistance or poor efficacy is a common problem of tyrosine kinase inhibitors in CML. Because many cases of resistance to tyrosine kinase inhibitors can be acquired through the activation of other tyrosine kinases,[51] growing attention has been focused on developing combinational approaches to interrupt different pathways. Because HDACi was reported to facilitate the ability of small molecule tyrosine kinase inhibitors,[47] we therefore examined the combinational effect of SAHA and S116836. KBM5 or KBM5-T315I cells were exposed to sublethal concentrations of SAHA and S116836 for 24 h, western blotting analysis showed that an increase in acetylation in histone H3 and H4, and representative molecule p53 in SAHA-treated or SAHA plus S116836-treated CML cells (**Fig. 2**). The phosphorylation of Bcr-Abl and its downstream molecules (e.g., STAT5 and Akt) was completely blocked in S116836-treated or SAHA plus S116836-treated CML cells (**Fig. 2A**). No alteration of the levels of Bcr-Abl was noted. Of interest, further decreases in the phosphorylated Erk1/2 levels were observed in SAHA plus S116836-treated CML cells when compared with each drug alone (**Fig. 2A**), suggesting that SAHA may be involved in the inactivation of Erk1/2. Additionally, the levels of STAT5 and Akt were dramatically reduced in the combination-treated CML cells in comparison with the single drug treated CML cells (**Fig. 2A**). The acetylation in histone H3 and H4 and acetylated p53 were increased in SAHA-treated or SAHA plus S116836-treated CML cells (**Fig. 2B**). Thus, it appears that the combination blocked the Bcr-Abl signaling and pro-survival signaling molecules.

SAHA and S116836 are synergistic to inhibit cell proliferation of imatinib-resistant CML cells

We next examined the combinational effect of SAHA, a broad spectrum of HDAC inhibitor, and S116836. We discovered that co-treatment of S116836 and SAHA synergistically reduced the cell growth of KBM5 and KBM-T315I cells when compared with SAHA or S116836 alone (**Fig. 3A**). The synergistic suppression of SAHA and S116836 on the proliferation capacity of KBM5 and KBM-T315I cells was further confirmed by colony formation assay. Although S116836 itself was able to lower the cell proliferative activity, SAHA greatly augmented its capacity in reducing anchorage-independent growth (**Fig. 3B**).

We profiled the cell-cycle distribution of CML cells treated with SAHA and S116836 (**Fig. 3B**). SAHA alone had a limited effect on the cell cycle of KBM5 and KBM5-T315I cells. S116836 for 24 h induced a pronounced decline in the S phase proportion and an accumulation of the G_1 phase proportion in imatinib-resistant KBM5-T315I cells. However, addition of SAHA to S116836 culture attenuated the change of G_1 and S phase

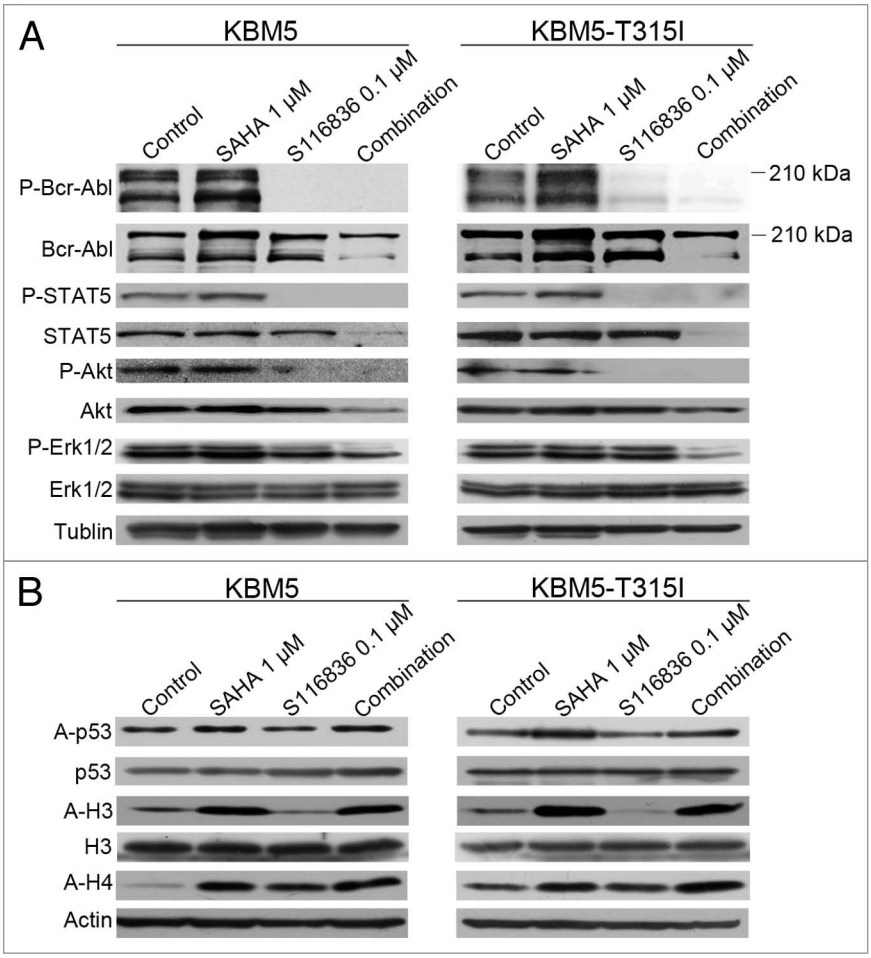

Figure 2. Co-treatment with SAHA and S116836 exerts enhanced activity in blocking downstream signaling of Bcr-Abl. (**A**) KBM5 or KBM5-T315I cells were cultured with histone deacetylases inhibitor suberoylanilide hydroxamic acid (SAHA) (1 μM), alone or in combination with S116836 (0.1 μM) for 24 h, after which cell lysates were subjected to western blotting analysis. Co-treatment reduces the expression and the phosphorylation of Bcr-Abl, STAT5, Akt, and Erk 1/2. (**B**) Western blotting analysis was done to monitor the levels of acetylated (acetyl)-histone 3, acetyl-H4 and acetyl-p53/p53 in the cell lysates from KBM5 or KBM5-T315I cells after the cells were treated with SAHA, plus S116836 or not for 24 h.

proportion in KBM5-T315I cells. SAHA induced significant increase in apoptosis in both KBM5 and KBM-T315I cells. These data suggest that SAHA may interrupt the cell cycle profile in imatinib-sensitive and imatinib-resistant CML cells.

SAHA enhances S116836-induced lethality in imatinib-sensitive and imatinib-resistant CML cells

We next explored the apoptosis in CML cells exposed to SAHA and S116836. KBM5 and KBM5-T315I cells were treated with the indicated concentrations of S116836 and SAHA alone or in combination for 24 h. The apoptotic cells were stained with Annexin V/PI, and detected by the flow cytometry. As shown in **Figure 3A**, SAHA (0 μM–1 μM) exhibited no toxicity to CML cells, and S116836 (0 μM–0.1 μM) induced minimal lethality either. However, when KBM5 cells or KBM5-T315I cells were exposed to combinational treatment, a substantial amount of apoptotic cells were observed (**Fig. 4A and B**). It's worth noting that the proapoptotic effect was much more significant in KBM5-T315I cells than that in KBM5 cells, indicating the combinational treatment may work in a Bcr-Abl-independent manner.

To draw a rigorous conclusion of synergistic effect, we used the widely-accepted CalcuSyn software to assess. The combination index (CI) < 1, = 1, and > 1, represent synergistic, additive, and antagonistic effects, respectively. Median dose effect analysis of apoptosis over various concentrations of S116836 and SAHA showed that the CI values of KBM5 cells were 0.126, 0.138, 0.167, and 0.168. The CI values of KBM5-T315I cells were 0.526, 0.541, 0.666, and 0.845 (**Fig. 4C**), indicating a synergism between SAHA and S116836 (note: CI < 1.0 indicates synergism).

Additional attempts were made to define the pathway of apoptosis. We employed separate or combined treatments of SAHA (1 μM) and S116836 (0.1 μM) to KBM5 or KBM5-T315I cells, and monitored the cytosolic fractions of cytochrome *c* by western blotting analysis. Although the individual effects of either SAHA or S116836 were minimal, the combination induced a striking increase in cytosolic cytochrome *c* (**Fig. 4D**). In accordance with the cytochrome *c* release, a considerable increases in cleavage of PARP procaspase-9, and pro-caspase-3 were observed. A substantial elevation in the levels of activated caspase-3 was also detected by western blotting analysis of the total cell lysate (**Fig. 4D**). Furthermore, the combination of the two drugs also elicited an enhanced cleavage of pro-caspase-8, revealing a synergistic effect to extrinsic apoptotic pathway.

The combinatory effect of SAHA and S116836 were then examined in relation to changes on various proteins involved in apoptosis. As shown in **Figure 4E**, individual or combinatory treatment did not alter the expressions of Bax and Bcl-X_L in both cell lines. In striking contrast, the levels of Mcl-1 and XIAP were dramatically reduced after co-treatment with SAHA and S116836 when compared with alone. In addition, the combined treatments led to a considerable upregulation of the pro-apoptosis protein Bim in both cell lines (**Fig. 4E**). S116836 alone or combined with SAHA induced a slight or modest decrease in Bcl-2 expression in KBM5 but not KBM5-T315I cells. Taken together, the combination of SAHA and S116836 disturbs the expression of Bcl-2 family members.

SAHA/S116836 regimens synergistically inhibits the growth of primary CML cells

The findings of synergistic effects of SAHA and S116836 suggest a potential combination of two therapeutic agents against CML. In an attempt to translate our observations into a clinical model system, we investigated the effects of co-treatment with

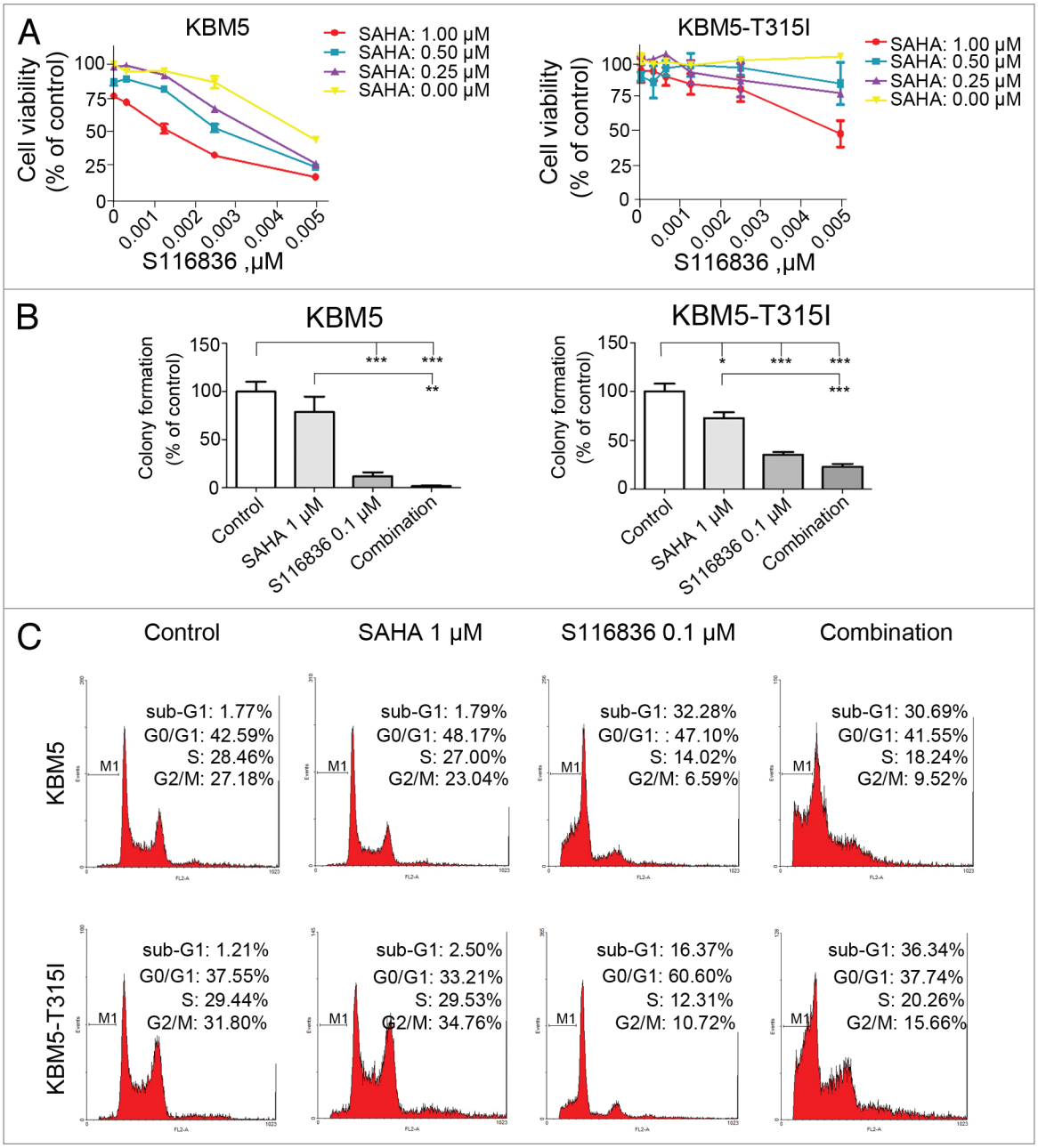

Figure 3. SAHA augments the activity of S116836 in inhibiting cell viability, colony formation, and inducing cell cycle disruptions. (**A**) After treatment with various concentrations of S116836 in the absence or presence of suberoylanilide hydroxamic acid (SAHA) for 72 h, viability of KBM5 or KBM-T315I cells was evaluated using the MTS assay. (**B**) KBM5 or KBM-T315I cells were exposed with SAHA, S116836 or the combination for 24 h, then the drugs were washed, the cells were seeded in soft agar for colony growth. The number of colony-forming in the absence of drug treatments (control) was defined as 100%. Then the yields of colony from the drug-treated cells were normalized relative to control. The experiments were independently repeated twice. A statistically significant difference was observed. Results are means with SEM; $^{**}P < 0.01$; $^{***}P < 0.0001$, one-way ANOVA, post hoc comparisons, Tukey test. (**C**) KBM5 or KBM-T315I cells were subjected to treatments of SAHA (1 μM) and S116836 (100 nM) alone or in combination for 24 h, after which flow cytometric analysis of the DNA content was performed to assess cell cycle distribution.

both agents on the growth of primary cells from patients with CML. The primary cells isolated from the peripheral blood or bone marrow from 5 patients with CML (**Table 1**) were treated with drugs with indicated concentrations for 72 h, the cell viability monitored by MTS assay revealed an inhibition of S116836 on the growth of CML cells (**Fig. 5A**), and addition of SAHA enhanced this inhibition. The CI values of these 5 patients was calculated by using the CalcuSyn software further supported a synergistic effect of the combination of SAHA and S116836 on the growth of primary CML cells (**Fig. 5B**).

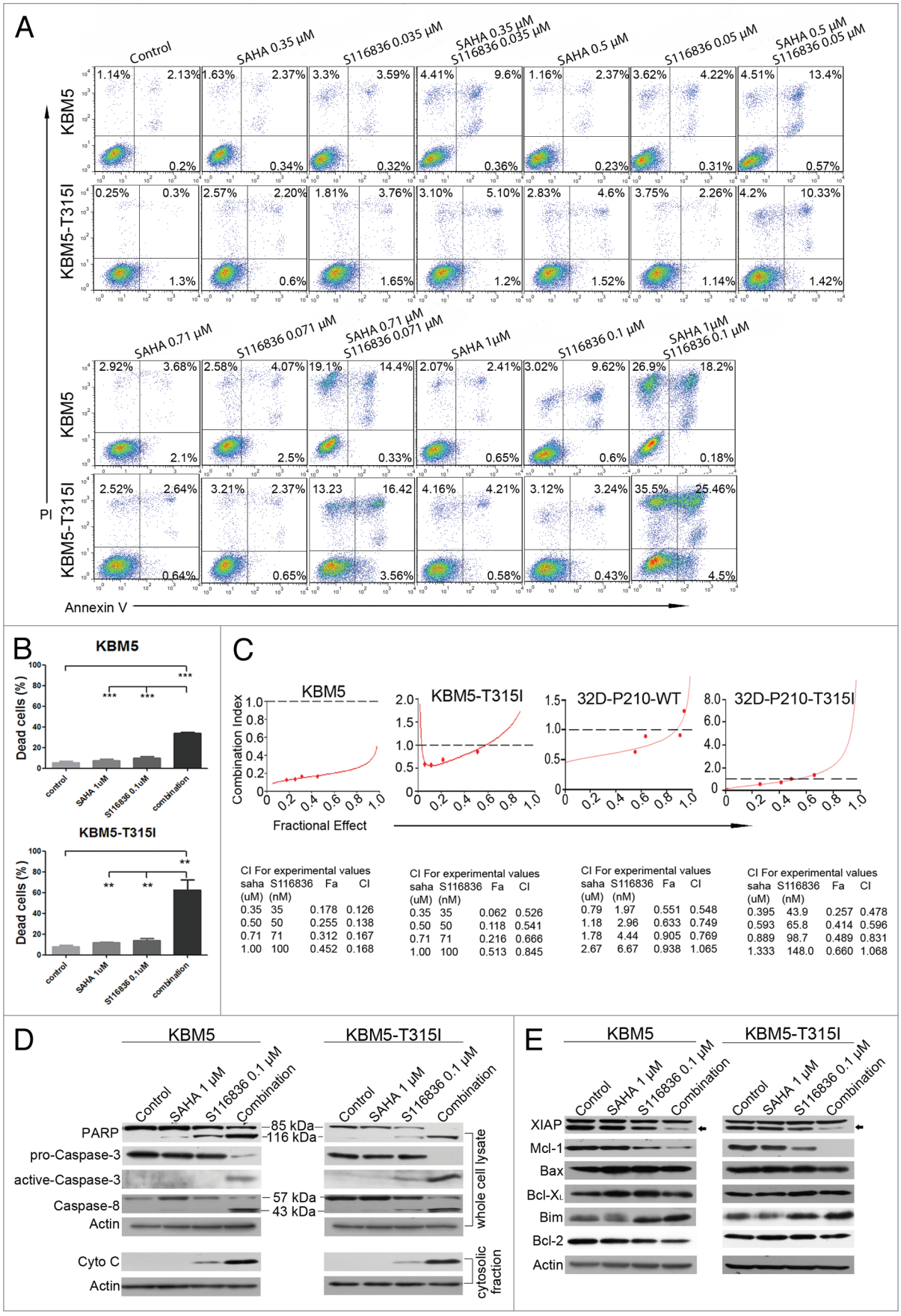

Figure 4. For figure legend, see page 957.

Figure 4 (See opposite page). Co-treatment of SAHA and S116836 leads to significant apoptosis in CML cells. (**A and B**) KBM5 or KBM-T315I cells were treated with various concentrations of S116836 in combination with different concentrations of SAHA for 24 h, after which they were stained with FITC-Annexin V/propidium iodide. The percentages of the apoptosis cells were determined by the flow cytometry (**A**). Quantitative analysis of dead cells in 3 independent experiments was shown (**B**). Columns represent triple respective experiments and the bars denote means with SEM. (**C**) KBM5 and KBM-T315I cells were treated with various doses of SAHA (0.31 μM~1 μM), S116836 (0.031 μM~0.1 μM), or combination of the 2 drugs. Twenty-four hours later, apoptosis was measured by flow cytometry. Combination index (CI) was analyzed by using the CalcuSyn Software. CI < 1 represents synergism. (**D**) The CML cells were co-treated with SAHA (1 μM) and S116836 (0.1 μM) for 24 h. The cells were divided into two portions, the first portion was prepared the whole cell lysate with RIPA buffer for western blotting of the cleavage of PARP and the levels of caspase-8, pro-caspase-3, and active caspase-3 (upper). The second portion of the cells was prepared the mitochondria-free cytosolic fractions to monitor the levels of cytochrome *c* by western blotting (lower). (**E**) The expression of apoptosis-related proteins in whole cell lysates was detected by western blotting analysis. Actin was used as a loading control.

Table 1. Characteristics of patients with chronic myelogenous leukemia

Patient no.	Age (y)/ sex	Date of diagnosis	Prior therapy	Date imatinib therapy began	WBC count (10^9/L)	Bcr-Abl positive	Bcr-Abl-Abl
1	32/M	11/2010	No treatment	11/2010	129	Yes	80%
2	33/M	11/2010	No treatment	12/2010	208.8	Yes	80%
3	28/M	3/2011	Hydroxyurea	3/2012	46.6	Yes	82.36%
4	55/M	2/2014	No treatment	3/2014	152.8	Yes	80.5%
5	23/F	3/2014	No treatment	3/2014	188.8	Yes	80%

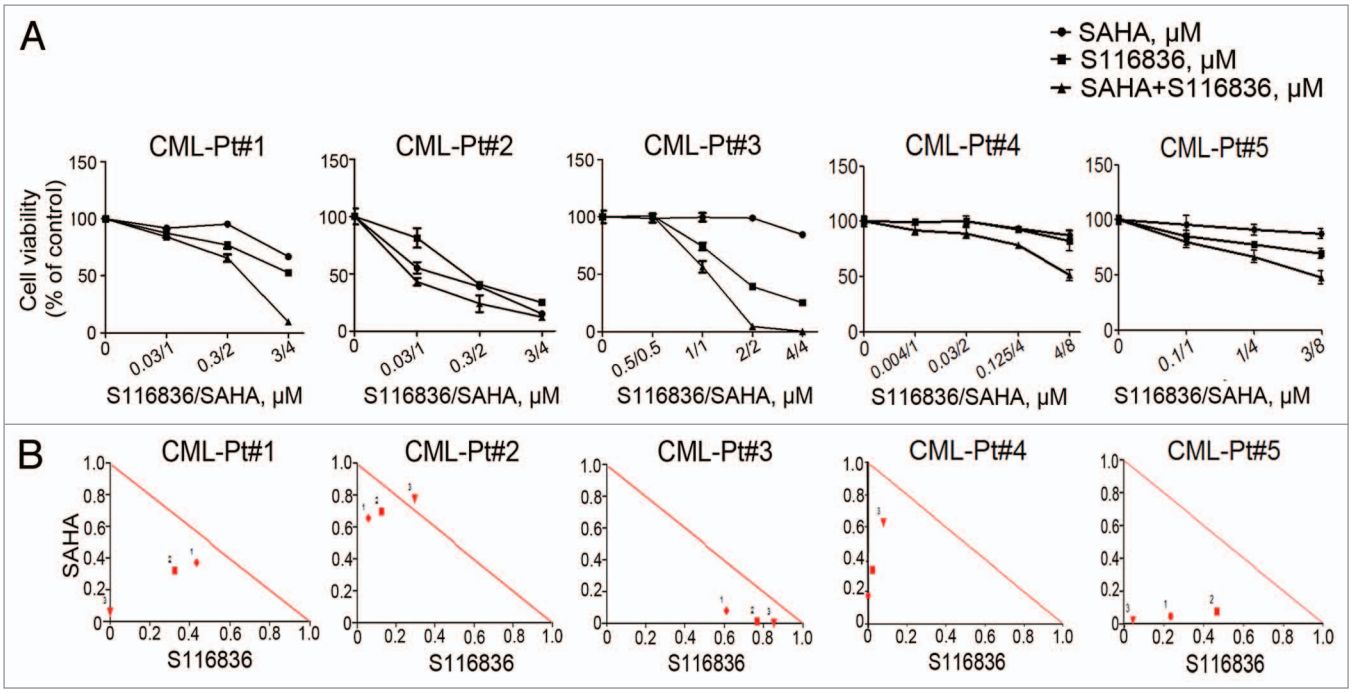

Figure 5. The combination of SAHA and S116836 synergistically inhibits the cell viability in primary CML cells. (**A**) Peripheral blood mononuclear cells from 5 CML patients were treated with various concentrations of SAHA in the presence or absence of S116836 for 72 h, after which the cell viability of each agent alone and of combination was analyzed by the MTS assay. (**B**) The combination index (CI) about the viability of the cells treated with SAHA and S116836 was calculated with CalcuSyn Software.

Discussion

Despite the success of imatinib in Bcr-Abl-positive CML,[52] the development of resistance to IM and dasatinib, especially for those in accelerated and blast phases, remains a major challenge for treatments.[13,15,53] A promising approach to tackle the challenge is combining targeted drugs to interrupt multiple signaling pathways simultaneously. In this study, we demonstrate that combination of the multi-targeted tyrosine kinase inhibitor S116836 and HDACi SAHA causes significant mitochondrial damage and apoptosis in IM-sensitive or -resistant CML cells. Our data show that using either agent alone causes none or very minimum apoptosis; however, combining these two drugs together induces an enhanced lethalty.

Our results were consistent with the findings of Fiskus et al., who reported that dasatinib and SAHA have synergism to kill imatinib-resistant cells.[47] However, S116836 is a novel compound that is active against T315I Bcr-Abl. Based on the median-effect

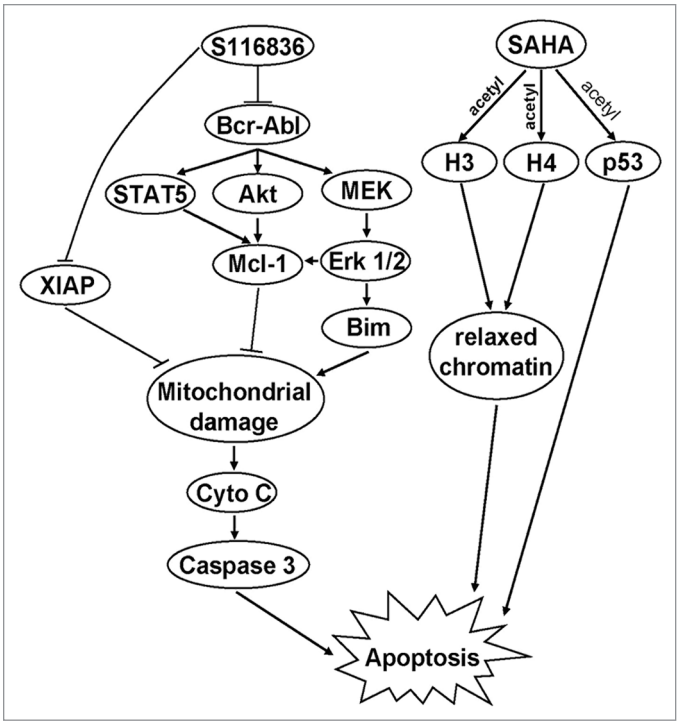

Figure 6. The proposed model of SAHA and S116836 to mediate apoptosis in CML cells. Co-treatment with SAHA and S116836 inhibits the activity of Bcr-Abl, Akt, Erk 1/2, and STAT5, which result in reduction of Mcl-1 and XIAP, and upregulation of Bim. These effects may contribute to mitochondrial damage and subsequently induce the apoptosis of imatinib-sensitive or resistant cells.

method of Chou and Talalay, we compared the synergistic effect of TKI and SAHA in a pair of human chronic myelogenous leukemia (CML) patient-derived imatinib-sensitive KBM5 cells expressing the 210 kDa native Bcr-Abl and imatinib-resistant KBM5-T315I cells harboring a threonine-to-isoleucine substitution at position 315 of Abl. In addition, the synergism was observed with a pair of 32D myeloid cells stably expressing either 210 kDa wild-type Bcr-Abl or T315I Bcr-Abl was the synergistic effect of TKI and SAHA.

The dual targeting strategy has been witnessed widely in many different types of cancers. A recent research in lung cancer model showed that interrupting a single pathway failed to induce cell death, and instead suppressing multiple pathways was essential to induce cell death as a consequence of inhibiting overlapping or redundant pathways.[54] In the case of acute myeloid leukemia (AML), Gilliland and Griffin have laid out a two-hit concept of leukemogenesis which serves as a theoretical basis for combining targeted agents for AML therapy. This theory posits that leukemogenesis acts through collaboration between two types of proteins: Type I, their dysfunction leads to disarranged cell differentiation; Type II, their dysfunction promotes cell survival.[55] Thus, in the present study, one potential mechanism underlying this S116836/SAHA-mediated apoptosis may be simultaneous suppressions on the dysregulated differentiation pathway and cell survival pathway. It is also possible that disrupting these pathways lowers the threshold for SAHA-mediated cell apoptosis. The precise mechanisms need to be further studied.

In our study, one key protein with a role in the synergistic process is Mcl-1. Inoue et al. has reported that HDACi mediated Mcl-1 upregulation and bring out the limited efficacy of apoptosis.[56] However, either co-administration with a Mcl-1 inhibitor or downregulation of Mcl-1 potentiates HDACi-mediated apoptosis in CML cells and CLL (chronic lymphocytic leukemia) cells.[56] SAHA will may upregulate Mcl-1 in CML cells, although our data show SAHA has no apparent effect on CML cells. S116836 has a modest effect in inhibiting Mcl-1. Thus, the synergistic effect of SAHA and S116836 may also work through the repression of Mcl-1 by S116836 and offset the side effect cause by SAHA.

While further studies are warranted to determine the mechanism by which S116836 regulate Mcl-1, several different pathways have been documented to modulate the expression of Mcl-1, including PI3K/Akt, STAT5, and MEK/ERK. PI3K/Akt regulates the Mcl-1 through a transcription factor complex containing CREB.[57] In CLL, sustained activation of Akt led to the increased expression of Mcl-1, Bcl-X_L, and XIAP, greatly increasing leukemic cell viability.[58] Our results showed that S116836 attenuated the phosphorylation of Akt, and addition of SAHA reduced not only the activity but also the total expression of Akt. The expression level of total Akt is consistent with that of Mcl-1. Therefore, a potential mechanism may be that, when treated with SAHA and S116836 together, downregulation of Akt contributes to the reduction of Mcl-1, and further leads to the synergistic effect of apoptosis. In addition to Akt, recent evidence suggests STAT5 may also play an crucial role in regulating the expression of Mcl-1.[59] In Bcr-Abl$^+$ cells, abnormal activation of STAT5 elevated the expression of Mcl-1, while silencing of STAT5 resulted in a decrease in the expression level of Mcl-1.[7,60] In our results, expression and activation of STAT5 is similar to Akt and Mcl-1, suggesting that STAT may also play a role in the regulation of Mcl-1. We also observed that combined treatment with SAHA and S118936 led to a reduction of activated Erk1/2, which is related to downregulation of Mcl-1. It has been reported that actived Erk1/2 stabilizes Mcl-1 through phosporylating its PEST region.[61] Sorafenib directly inhibited Erk1/2 to lower the expression of Mcl-1.[62]

In addition, XIAP and Bim also showed synergism as a result of SAHA/S116836 combined treatments. We speculate that all of these proteins contribute to the induction of mitochondrial damage, which is reflected on the increasing release of cytochrome *c* and the PARP cleavage. In summary, our finding uncovered a new strategy of dual treatments that combining the S116836 and SAHA significantly induces apoptosis in CML cells. This synergistic effect may involve multiple mechanisms shown in **Figure 6**.

It is worthy to note that enhanced lethality of SAHA/S116836 was not only seen in IM-sensitive cells, but also in the resistant ones. Several mechanisms often associated with IM resistance consist of Bcr-Abl overexpression, reduced uptake of IM, and acquisition of point mutation (e.g., T315I) in the Bcr-Abl kinase

domain known to be essential for IM binding.[63] As showed in our results, S116836 is a potent compound in preventing the activation of Bcr-Abl no matter Bcr-Abl bears a T315I mutation or not. However, the effect of S116836 in inducing apoptosis of KBM5 or KBM5-T315I cells is still mild, indicating that, apart from the inhibition of Bcr-Abl, other mechanisms may have a role in the synergistic effect. Donato et al. found undetectable expression of BCR-ABL protein in imabinib-resistant cells from CML patients, suggesting that IM resistance may stem from a Bcr-Abl-independent manner.[64] Some other aberrantly expressed proteins beside Bcr-Abl may also cause this resistance in IM-resistant CML. Although Src-related Lyn kinase and Erk1/2 has been demonstrated that associated with the Bcr-Abl-independent resistance,[65,66] it is still unclear whether any other proteins participate in this resistance. In this context, a multi-targets therapy is an ideal approach.

Although the precise mechanisms responsible for this phenomenon are elusive, HDACi has been well accepted to make leukemia cells more susceptible to apoptosis, and there are several successful examples of combining tyrosine kinase inhibitors with HDACi to induce apoptosis of CML cells resistant to IM—for instance, SAHA and IM, SAHA and dasatinib, LBH589 and AMN107.[47,67,68] Here we present the combination of SAHA and S116836 as a new therapy for treating IM-resistant CML.

Materials and Methods

Chemicals and antibodies

S116836 (structure shown in **Fig. 1A**) was dissolved in DMSO and 20 mM stock solution was stored at −20 °C. Antibodies against c-Abl (C-19), p53 (DO-7), Mcl-1 (S-19), Bcl-X_L, histone H3, caspase-3, and Bax were from Santa Cruz Biotechnology. Antibodies against poly (ADP)-ribose polymerase (PARP; clone 4C10-5), cytochrome *c*, XIAP were from BD Biosciences. Antibodies against phospho-c-Abl (Y245), phospho-Erk 1/2 (T202/Y204), Erk 1/2, Akt, acetyl-histone H3 (K9), acetyl-histone H4 (K16), acetyl-p53 (K382), and caspase-8 were from Cell Signaling Technology. Antibodies against phospho-STAT5, STAT5, and Bcl-2 were from EMD Millipore Upstate.

Cell culture

KBM5 cells bearing 210 kDa wild-type Bcr-Abl were sensitive to IM. KBM5-T315I cells bearing T315I mutation in Bcr-Abl have the resistance to IM. Both of these cell lines were cultured in IMDM (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS), as described previously.[69] In addition, KBM5-T315I cells were also grown with 1.0 μM IM. imatinib was removed before experiments followed by a wash-out period of 2–3 d. The 32D myeloid cells stably expressing either 210 kDa wild-type Bcr-Abl (32D-P210-WT) or T315I Bcr-Abl (32D-P210-T315I) were maintained in RPMI 1640 with 10% FCS. Peripheral blood cells were obtained from 5 CML patients in the First Affiliated Hospital and Guangdong Provincial People's Hospital (**Table 1**). The research is consistent with the institutional guidelines and the Declaration of Helsinki principles. The isolation of mononuclear cells has been described in previous work.[70,71] Cells were suspended in RPMI 1640 supplemented with 10% FCS.

Cell viability assay

Cell viability was determined by MTS assay (CellTiter 96 Aqueous One Solution reagent; Promega).[70,71] Cells were plated in quadruplicate onto the 96-well plates, and treated with the escalated concentrations of drugs for 72 h before performing the MTS assay. The optical density (O.D.) values for untreated group were set as 100% viability; the values of treated groups were normalized against the control group. The drug concentration that induced 50% inhibition of cell growth (IC_{50}) was calculated by regression fitting of a dose-response curve.

Flow cytometry analysis of cell cycle

Cells were exposed to various concentrations of drugs, and then the cells were collected and fixed in 66% (V/V) cold ethanol at −20 °C. After more than 16 h, cell were washed twice with cold PBS and suspended with propidium iodide and RNase A (0.5 mg/mL) (in the dark) for 1 h. Data of cell cycle distribution was collected by using FACSCalibur flow cytometer and CellQuestPro software.

Clonogenicity assay

Cells treated with the indicated concentrations of SAHA and S116836 for 24 h, were then collected and washed twice with PBS, then, cultured in drug-free Iscove's medium containing 0.3% agar and 20% FCS. After the ~10 d incubation, the number of colonies was counted.

Assessment of apoptosis

Apoptosis was evaluated by using of Annexin V/propidium iodide (PI) binding assay according to the instruction of the manufacturer (Sigma-Aldrich). Samples were analyzed by using of FACSCalibur flow cytometer and CellQuestPro software as previously described.[72,73]

Western blotting analysis

Western analyses were performed as previously described.[74]

Statistical analysis

Experiments were performed at least three times. GraphPad Prism 5.0 software (GraphPad Software) was used for statistical analysis. Combination Index was calculated by CalcuSyn software 2.0 according to the software's instruction.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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